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Gene silencing in root lesion nematodes (*Pratylenchus* spp.) significantly reduces reproduction in a plant host

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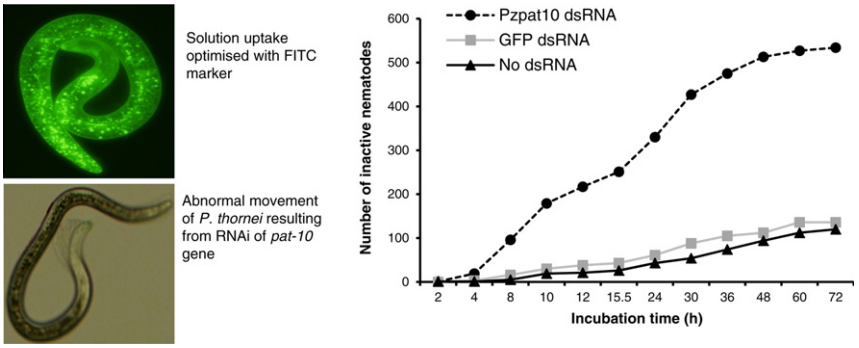
HIGHLIGHTS

- *P. thornei* and *P. zeae* are amenable to double stranded RNA-induced gene silencing via soaking.
- Using spermidine phosphate salt hexahydrate in soaking media results in more effective gene silencing.
- Silencing *pat-10* and *unc-87* of *P. thornei* reduces reproduction by 77–81% in carrot mini discs.
- Double stranded RNA from either nematode species silenced the corresponding gene in both species.
- RNA interference is demonstrated to be a potential control strategy for root lesion nematodes.

GRAPHICAL ABSTRACT



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ABSTRACT

Root lesion nematodes (RLNs, *Pratylenchus* species) are a group of economically important migratory endoparasitic plant pathogens that attack host roots of major crops such as wheat and sugarcane, and can reduce crop yields by 7–15%. *Pratylenchus thornei* and *Pratylenchus zeae* were treated with double stranded RNA (dsRNA) to study gene silencing, (RNA interference, RNAi), as a potential strategy for their control. Mixed stages of nematodes of both species ingested dsRNA when incubated in a basic soaking solution in the presence of the neurostimulant octopamine. Incubation for up to 16 h in soaking solutions containing 10–50 mM octopamine, 0.1–1.0 mg/mL FITC, and 0.5–6 mM spermidine did not affect vitality. Spermidine phosphate salt hexahydrate rather than spermidine or spermidine trihydrochloride increased uptake of FITC by nematodes, and this resulted in more effective gene silencing. Silencing *pat-10* and *unc-87* genes of *P. thornei* and *P. zeae* resulted in paralysis and uncoordinated movements in both species, although to a higher degree in *P. thornei*. There was also a greater reduction in transcript of both genes in *P. thornei* indicating that it may be more susceptible to RNAi. For *P. thornei* treated with dsRNA of *pat-10* and *unc-87* there was a significant reduction (77–81%) in nematode reproduction on carrot mini discs over a 5 week period. The results show that RLNs are clearly amenable to gene silencing, and that *in planta* delivery of dsRNA to target genes in these nematodes should confer host resistance. Moreover, for the two genes, dsRNA derived from either nematode species silenced the corresponding gene in both species. This implies cross-species control of nematodes via RNAi is possible.

Abbreviations: PPN, plant parasitic nematode; RLN, root lesion nematode; RNAi, RNA interference; dsRNA, double stranded RNA; FITC, fluorescein isothiocyanate.

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1. Introduction

Root lesion nematodes (*Pratylenchus* spp.) are economically important migratory endoparasitic pests of agricultural, horticultural and industrial crops (Oliviera et al., 1999; Seinhorst, 1998; Smiley et al., 2005). Both juvenile and adult nematodes can enter and leave root tissues, move between cortical cells and feed from them: their life cycle takes 3–8 weeks to complete (Castillo and Vovlas, 2008). Feeding by *Pratylenchus* spp. deprives host plants of essential nutrients, whilst entry wounds make plant roots more susceptible to bacterial and fungal pathogens present in the soil. The focus of control strategies for these and other nematodes has depended on application of expensive and environmentally unfriendly chemicals, deployment of natural resistance genes and cultural practices. The current emphasis is to move to nematode-centred environmentally friendly approaches. One such strategy involves the application of RNA interference (RNAi), first to understand the function of genes essential to nematode development and parasitism and second to confer resistance to host plants engineered to express dsRNA and small interfering RNAs (siRNA) to target and silence specific nematode genes.

The discovery of RNAi in *Caenorhabditis elegans*, in which double stranded RNA (dsRNA) induces the degradation of cognate endogenous mRNA and so prevents synthesis of the encoded protein, has provided a significant new tool to study gene function (Mello and Fire, 1995). RNAi has been investigated in many organisms including mammals, insects, fungi and plants (Bucher et al., 2002; Elbashir et al., 2001; Kennerdell and Carthew, 1998; Ngô et al., 1998; Romano and Macino, 1992). In *C. elegans*, RNAi can be triggered by exogenous dsRNA (100–500 bp long) delivered via soaking in buffered-solutions, by microinjection of dsRNA into the adult worm or by feeding on *Escherichia coli* engineered to produce dsRNA (Fire et al., 1998; Fraser et al., 2000; Tabara et al., 1998; Timmons and Fire, 1998). This technology, and sequencing of the genome of *C. elegans*, has enabled functional analysis of almost all of its genes, making it the best annotated multicellular organism (*C. elegans* Sequencing Consortium, 1998, www. wormbase.org).

RNAi now provides new opportunities for research on plant parasitic nematodes (PPNs). Delivery of dsRNA to nematode juveniles via 'soaking' in solutions containing dsRNA has been used successfully to investigate the function of some genes in cyst nematodes (e.g. *Globodera pallida* and *Heterodera glycines*), root knot nematodes (e.g. *Meloidogyne incognita*, *Meloidogyne hapla* and *Meloidogyne javanica*) and migratory nematodes such as *Radopholus similis* and *Bursaphelenchus xylophilus* (Adam et al., 2008; Cheng et al., 2010; Haegeman et al., 2009; Huang et al., 2006; Park et al., 2008; Rosso et al., 2005; Urwin et al., 2002). For these obligate parasites, uptake of exogenous dsRNA is enhanced by neurostimulants such as octopamine, resorcinol or serotonin in the soaking solution (Bakhetia et al., 2005; Dubreuil et al., 2007; Rosso et al., 2005). Uptake of soaking solution containing dsRNA in the stylet, pharynx and intestinal tract can be been monitored either with fluorescently labelled dsRNA or the fluorescent dye, fluorescein isothiocyanate (FITC) as a marker, which can be included with dsRNA in soaking solutions. Appropriate controls are needed because adverse effects on nematodes have occasionally been reported from FITC and some components of the soaking solution (Adam et al., 2008; Huang et al., 2006; Rosso et al., 2005; Schroeder and MacGuidwin, 2007; Sukno et al., 2007; Urwin et al., 2002). There is now good evidence that RNAi can be used as a control strategy for PPNs in that *in planta* delivery of siRNA/dsRNA reduces nematode establishment and development (Fairbairn et al., 2007; Huang et al., 2006; Sindhu et al., 2009; Yadav et al., 2006). However, there has been no published work to show whether RLNs are also amenable to RNAi.

Until recently, RLNs were relatively neglected pests of crop plants, but with the availability of transcriptome data for some of these species, research on host interactions leading to potential control can be pursued more readily (Haegeman et al., 2011; Nicol et al., 2012). Unlike other PPNs, for which several of their *in vitro*, axenic culture and experimental hosts can be genetically transformed relatively easily, suitable experimental systems are not readily available for root lesion nematodes. Most migratory endoparasitic nematodes have been cultured on alfalfa callus, but monoxenic cultures of root lesion and burrowing nematodes on callusing carrot discs provide a convenient culture system (Kaplan and Davis, 1990; Krusberg, 1961; Lownsbery et al., 1967; Reise et al., 1987).

In this study, the efficacy of RNAi in *Pratylenchus thornei* and *Pratylenchus zeae* has been studied using soaking to introduce dsRNA. Possible effects of different components of the soaking solution on the activity of nematodes have been examined. To investigate long-term effects of RNAi on *P. thornei*, the carrot disc technique was adapted to culture and extract RLNs for higher throughput experimentation, using mini discs in 24 well plates, for which only 50 nematodes were used as an initial inoculum on each disc. This system was used to assess how silencing calponin and troponin C genes delivered by soaking nematodes in dsRNA affected establishment and reproduction of *P. thornei*. In *C. elegans*, these two genes are required to maintain structure and contraction of muscles, and hence their orthologues in RLNs would be important genes in these migratory nematodes. Here we confirm that root lesion nematodes are amenable to gene silencing by RNAi, and that a significant reduction in nematode reproduction after soaking in dsRNA of two target genes was obtained.

2. Materials and methods

2.1. Identification of target genes

P. thornei and *P. zeae* orthologues of the *C. elegans* troponin C (*pat-10*) and calponin (*unc-87*) genes were used to test the amenability of these RLNs to RNAi. The genes were obtained using amino acid sequences of *C. elegans pat-10* (wormbase ID F54C1.7) and *unc-87* (wormbase ID F08B6.4c) to query several databases including the National Centre for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/), Nembase (www.nematode.org) and Nematode.net using TBLASTX 2.2.26+ (Altschul et al., 1997). Translated amino acid sequences of two ESTs from mixed stages of *Pratylenchus penetrans* with high identities and sequence coverage were selected: BQ627209 (557 nucleotides, nt) was 84% identical to *C. elegans pat-10* whereas BQ626831 (524 nt) was 80% identical to *C. elegans unc-87*. Primers Pppat-10F and Pppat-10R, and Ppunc-87F and Ppunc-87R, were designed based on sequences BQ627209 (NCBI) and BQ626831 (NCBI) to amplify the equivalent sequences in *P. thornei* and *P. zeae* using cDNAs generated from mixed stages of *P. thornei* and *P. zeae*. PCR products for both genes differed in sizes and are designated with the prefixes Pt or Pz for *P. thornei* and *P. zeae*, respectively (Table 1). Sequences of the ESTs have been deposited in GenBank: *Ptpat-10* (NCBI: JX122489), *Ptunc-87* (NCBI: JX122490), *Pzpat-10* (NCBI: JX122491) and *Pzunc-87* (NCBI: JX122492).

2.2. RNA extraction, reverse transcription and PCR amplification of target genes

RNA used for both reverse transcription-polymerase chain reaction (RT-PCR) of target genes and quantitative RT-PCR was extracted from mixed stages populations of *P. thornei* and *P. zeae* using TRIzol® Reagent (Life Technologies Corporation) and ethanol precipitation. The number of nematodes used depended on the purpose of the experiment. Nematodes were first macerated with